DEVELOPMENTAL BIOLOGY **180,** 156–164 (1996)
ARTICLE NO. 0292

The Inhibition of Differentiation Caused by TGFβ Expression of PKCθ: A Possible Molecular Basis for Myoblast Diversification during Limb Histogenesis

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Embryonic and fetal skeletal myoblasts are responsible for the formation of primary and secondary fibers in mammals, but the mechanism which diversifies their fate is unknown. *In vitro,* embryonic myoblasts are resistant to the differentiation inhibitory effects of transforming growth factor β and phorbol esters. Thus, differential expression of specific molecules involved in the transduction of extracellular signals may contribute to the different phenotypes. We report here that protein kinase C θ, but none of the other known protein kinase C isoforms, is selectively expressed in fetal and postnatal muscle cells (at both the myoblast and myotube stage) *in vitro* and *in vivo*. By contrast, embryonic myoblasts and myotubes do not express protein kinase C θ *in vitro* or *in vivo*. This difference is causally related to a differential response to transforming growth factor β, since overexpression of protein kinase C θ, but not of protein kinase C α or ζ, in embryonic myoblasts makes these cells sensitive to transforming growth factor β. These data demonstrate for the first time that a protein kinase C isoform is a key component of the signal transduction cascade which follows exposure of myoblasts to transforming growth factor β. They also suggest a specific role for protein kinase C θ in determining the fate of different myoblasts during muscle histogenesis.

INTRODUCTION

During the histogenesis of murine skeletal muscle, two classes of myoblasts, respectively termed embryonic and fetal, populate the future muscle primordia and give rise asynchronously to primary and secondary muscle fibers. Although it has never been formally proven, it is generally assumed that embryonic myoblasts differentiate into primary fibers during the embryonic period of development, while fetal myoblasts do not participate in primary fiber formation and, after a period of active proliferation, differentiate mainly into secondary fibers in the fetus (Cossu and Molinaro, 1987; Harris *et al.,* 1989; Stockdale, 1989; Cossu *et al.,* 1992). At this time, satellite cells are first recognized as a separate myogenic cell type, responsible for postnatal fiber growth and regeneration (Cossu and Molinaro, 1987; Stockdale, 1989; Cossu *et al.,* 1992).

In vitro studies on muscle cell lines have shown that myoblasts proliferate in the presence of mitogens (mainly fibroblast growth factors, FGFs) and differentiate in their absence (Clegg *et al.,* 1987). However, this simple model cannot be applied to developing muscle *in vivo* where differentiating and dividing myoblasts coexist within the same microenvironment. Recent *in situ* and organ culture studies have shown that very high levels of FGF-4 are expressed in the apical ectodermal ridge (AER) (Niswander and Martin, 1992; Drucker and Golfarb, 1993) and that FGF-4 can promote limb bud growth even in the absence of the AER (Niswander and Martin, 1993). These findings suggest that a high concentration of mitogens exists in the distal region

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0012-1606/96 $18.00

of the growing limb. This may establish the conditions for the initial formation of primary fibers at the base of the limb (where mitogen concentration is likely to be lower). Alternatively, down-regulation of receptors for growth factors has been proposed as a key event in the initial muscle differentiation in the limb (Itoh *et al.*, 1996). Neither of the two possibilities, however, explains why embryonic myoblasts differentiate while fetal myoblasts do not.

Recently, several different observations, reported by many laboratories, suggest that transforming growth factor β (TGFβ) may play a regulatory role in skeletal muscle histogenesis. First, TGFβ is abundant in the limb bud; it is produced by the ectoderm, and it probably acts in a paracrine fashion on adjacent mesenchymal cells (Heine *et al.*, 1987; Lehnert and Akhurst, 1988; Pelton *et al.*, 1989; Millan *et al.*, 1991; Schmid *et al.*, 1991). Second, embryonic myoblasts differentiate in the presence of TGFβ while fetal myoblasts do not (Schofield and Wolpert, 1990; Cusella-De Angelis *et al.*, 1994). Finally, treatment of limb bud organ cultures with anti-TGFβ neutralizing antibodies results in the earlier appearance of large fetal-type myotubes in culture (Cusella-De Angelis *et al.*, 1994). Currently, the molecular mechanisms underlying these phenomena are still unclear. It is possible that molecules involved in TGFβ signal transduction are differentially expressed and/or activated in the two populations of myoblasts.

Very little information is available regarding the TGFβ-induced signaling events. Most studies have been conducted in cells where TGFβ interferes with cell proliferation. The signaling mechanisms leading to changes in cell proliferation by TGFβ are likely to be different, however, in cells that are growth stimulated versus those that are growth inhibited. Moreover, the role of TGFβ in myoblast differentiation is quite different, since it inhibits myogenic differentiation without interfering with cell proliferation (Florini *et al.*, 1986; Massaguè *et al.*, 1986; Olson *et al.*, 1986). Taken together, these data indicate that TGFβ may be able to activate different intracellular signaling mechanisms depending upon the status or type of the cell (Mulder and Morris, 1992; for a review see Derynck, 1994).

One molecule that is often implicated in these signaling cascades and that appears to mediate different cellular processes, such as growth and differentiation, is protein kinase C (PKC). PKC constitutes a family of serine/threonine protein kinases, and, to date, 11 different isoforms have been identified and cloned (Nishizuka, 1992). These isoenzymes can be divided into three major groups: the conventional calcium- and phospholipid-dependent PKCs (cPKCα, β_1, β_2, and γ), the novel calcium-independent PKCs (nPKCδ, ϵ, η, and θ), and the atypical calcium- and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-independent isoforms (ζ, ι/λ, and μ) (for a review see Ono *et al.*, 1989; Nishizuka, 1992; Dekker and Parker, 1994; Johannes *et al.*, 1994). These different isoenzymes have been shown to exhibit differential tissue distribution and cellular localization. While the α, β, δ, and ζ isoenzymes are ubiquitous, many of the others (e.g., γ, η, and θ) are restricted to one or a few tissues (Osada *et al.*, 1992; Wetsel *et al.*, 1992). Multiple isoenzymes are often found in a single cell type (Wetsel *et al.*, 1992; Hug and Sarre, 1993). To date, very little is known about substrate specificity among the different isoforms, but data are currently being collected about specific biological roles in different systems (Mischak *et al.*, 1993b; Murray *et al.*, 1993; Dekker and Parker, 1994; Bouché *et al.*, 1995).

The PKCθ is a member of the nPKCs subfamily which is the predominant PKC isoenzyme expressed in skeletal muscle (Osada *et al.*, 1992; Mischak *et al.*, 1993a). PKCθ expression varies with muscle type (Donnelly *et al.*, 1994) and innervation (Hilgenberg and Miles, 1995). Although it is clear that PKCθ plays a crucial role in muscle signal transduction pathways, its mechanism of action is still obscure.

In this paper we analyze PKCθ expression during limb muscle development and show that this isoenzyme is expressed only in fetal muscle (17 days postcoitum, dpc). Furthermore, our results demonstrate that PKCθ is involved in the differential response to TGFβ exerted by the different cell populations of myoblasts.

MATERIALS AND METHODS

Cell culture. Muscle cells were cultured from limbs of mouse embryos (11 dpc) or fetuses (16 dpc) as previously described (Cossu *et al.*, 1988). The cells were grown in Dulbecco's minimum essential medium (DMEM, Hyclone) supplemented with 10% horse serum (HS; Hyclone) and 3% chick embryo extract (EE) for 3 days. Mouse satellite cells (MSC) were prepared (Cossu *et al.*, 1980, 1983) and grown in DMEM supplemented with 20% HS and 5% EE. To induce differentiation, the cells were shifted to DMEM supplemented with 5% HS and 1.25% EE for 3 days. COS1 cells were obtained from ATCC and cultured in DMEM containing 10% FCS.

At the indicated time, cultures were either fixed and stained with different antisera/antibodies or processed for preparation of cellular extracts.

Antisera and antibodies. Several different primary antisera were used in this study. The PKCθ antiserum was raised against the sequence C332 VPTGKKREPQGISWDSPLDGSNK355 of PKCθ and purified by affinity column (Osada *et al.*, 1992). It did not react with any other known PKC isoforms (Osada *et al.*, 1992). The PKCα antiserum was raised against amino acids 313–326 of the human isoenzyme (Boehringer-Mannheim). Antisera to PKCβ_1, β_2, γ, δ, ϵ, and ζ (Wetsel *et al.*, 1992; Bouché *et al.*, 1995) and η (W. C. Wetsel, unpublished) were raised in rabbits against unique amino acid sequences located in the extreme carboxyl terminals of the different enzymes (Wetsel *et al.*, 1992; Bouché *et al.*, 1995). A monoclonal antibody (mAb) to the myosin heavy chain (MF20 antibody), which recognizes all sarcomeric myosins, was used to determine the differentiation state of the cells (Bader *et al.*, 1982).

cDNA probes and expression vectors. The SRD plasmids, containing the full-length cDNAs coding for mouse PKCs θ (SRDθ), α (YK504), ζ (M246), and δ (M241) under SV40 promoter control, were used for expression studies (Osada *et al.*, 1992; Mizuno *et al.*, 1991). A 1350-bp fragment obtained by *Bam*HI/*Eco*RI digestion of the SRDθ plasmid and a 1000-bp fragment obtained by *Hin*dIII digestion of the GAPDH plasmid (obtained from ATCC) were used as cDNA probes.

Preparation of cellular and tissues extracts. Cell pellets were resuspended in ice-cold homogenization buffer (H-buffer) containing 20 m*M* Tris (pH 7.5), 2 m*M* EDTA, 2 m*M* EGTA, 250 m*M* sucrose, 5 m*M* DTT, 200 μg/ml leupeptin, 10 μg/ml Trasylol, 1 m*M* PMSF, and 0.1% Triton X-100 and disrupted by sonication. Total protein extracts were prepared from 13 or 17 dpc mouse embryonic limbs or postnatal muscle. The limbs were removed from mouse embryos, washed in ice-cold PBS solution, homogenized in ice-cold H-buffer, and sonicated. Postnatal skeletal muscle was dissected from the hind limbs of newborn or adult mice; tissues were homogenized in ice-cold H-buffer and sonicated. The homogenate was incubated for 30 min on ice with repeated vortexing and then centrifuged at 15,000*g* for 15 min. The pellet was discarded. An aliquot of the supernatant was used for protein determination according to the instructions included with the Coomassie Plus protein assay reagent (Pierce), while the remainder was used for Western blot analysis.

Western blot analysis. Total lysates were mixed with 4× Laemmli sample buffer and boiled for 5 min, and 50 μg of each sample was loaded onto 10% SDS–polyacrylamide gels (Laemmli, 1970). The gels were blotted onto nylon filter (Hybond-C Extra, Amersham) and probed with the appropriate specific antisera. Peroxidase-conjugated goat anti-rabbit IgG (Cappel) was used as secondary antibody and detection was performed by the ECL method (Amersham) according to the manufacturer's instructions.

Immunofluorescence analysis. The embryonic or fetal myotubes in culture were fixed 3 days after plating. Proliferating MSC were maintained in growing medium and fixed after 2 days in culture. MSC-derived myotubes were fixed after 3 days in culture in differentiative medium. Tissue sections were prepared as described (Cusella-De Angelis *et al.*, 1994).

The cultured cells and the tissue sections were incubated for 1 hr with the anti-PKCθ antiserum (diluted 1:100) together with the anti-myosin heavy-chain mAb MF20 (diluted 1:5) (Bader *et al.*, 1982). The samples were then incubated for 1 hr with a biotin-conjugated goat anti-rabbit IgG (diluted 1:1600, Sigma). Finally, the samples were coincubated for 1 hr with fluorescein-conjugated goat anti-mouse (diluted 1:200, Cappel), TRITC-conjugated extravidin (diluted 1:200, Sigma), and Oechst. Cultures and slides were mounted in Tris (pH 9.00) supplemented with 60% glycerol and photographed under an epifluorescence Zeiss microscope.

Northern blot analysis. Total RNA was isolated by the acid guanidinium–phenol–chloroform method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated from total RNA by oligo(dT)–cellulose columns (Collaborative Research) (Sambrook *et al.*, 1989). Ten micrograms of Poly(A)$^+$ RNA was electrophoresed through a 1.2% agarose–formaldehyde gel and transferred to nylon filters (Gene Screen Plus, Dupont). The hybridization was performed in 50% formamide, 10% dextran sulfate, 11.6 mg/ml NaCl, 0.1 mg/ml denatured salmon sperm DNA, and $1–3 \times 10^6$ cpm/ml of denatured random-primed ^{32}P-labeled cDNA probes at 42°C for 24 hr. The last washing was performed in 0.2× SSC, 0.1% SDS at 55°C and the blots were exposed to autoradiography film (Reflection, Dupont).

DNA transfection. Embryonic myoblasts from 11 dpc embryos were plated in 35-mm tissue culture dishes. Twenty-four hours after plating the cells, at 70% of confluence, were transfected with 5 μg of DNA by Transfection-reagent (DOTAP; Boehringer-Mannheim) for 24 hr. After transfection the cells were cultured for 2 days in the absence or presence of 5 ng/ml of TGFβ_1 or 100 n*M* TPA (both from Sigma) and fixed for immunofluorescence analysis.

COS1 cells were transfected with the SRDθ plasmid by electroporation as described (Levin *et al.*, 1992).

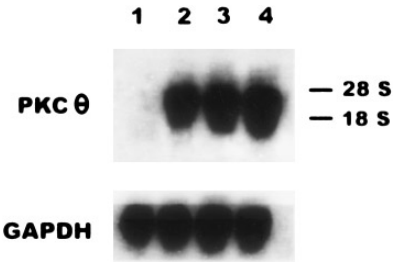


FIG. 1. Northern blot analysis of 13 dpc (lane 1) and 17 dpc (lane 2) embryo limbs or neonatal (lane 3) and adult (lane 4) skeletal muscle. Ten micrograms of poly(A)$^+$ RNA was loaded into each lane and hybridized with the ^{32}P-labeled PKCθ cDNA. The ^{32}P-labeled GAPDH cDNA was used for normalization.

RESULTS

PKCθ Is Differentially Expressed in Embryonic and Fetal Limb Muscle

To examine the possible role of PKCθ in muscle development and histogenesis, we first analyzed its RNA and protein expression during skeletal muscle development. Poly(A)$^+$ RNA was prepared from limb buds of mouse embryos at different stages of development and from postnatal skeletal muscle. Northern blot hybridization was performed using the PKCθ cDNA as a probe (Fig. 1). The accumulation of the PKCθ specific message is not detectable in either 13 dpc embryo limb buds (Fig. 1, lane 1) or limbs up to 15 dpc (not shown), while it is first evident at 17 dpc in the limb (Fig. 1, lane 2) and it is maintained at later stages in neonatal and adult skeletal muscle (Fig. 1, lanes 3 and 4). It is important to stress that mRNA for muscle-specific products is first detectable by this method between 10 and 12 dpc (see for example Barbieri *et al.*, 1990).

Western blot and immunofluorescence analyses confirmed the Northern blot data. Total protein lysates were prepared from developing and postnatal skeletal muscle, at the same stages used for the Northern blot, and examined using the specific anti-PKCθ antiserum. COS1 cells that overexpressed PKCθ were used as a positive control. The anti-PKCθ antiserum recognized a single polypeptide which migrated with a M_r of approximately 79×10^3 on SDS–PAGE (Fig. 2, lane 1); it does not cross-react with any of the other PKC isoforms, as already described (Osada *et al.*, 1992). Western blots from samples taken at different ages revealed that PKCθ becomes detectable, by this method, only in 17 dpc embryos (Fig. 2, lanes 3–5). There was no evidence for PKCθ protein expression at earlier stages (Fig. 2, lane 2).

These Northern and Western data were further confirmed by immunocytochemistry. Immunofluorescence analysis of cryosections of limb buds at 13 dpc revealed that the great majority of cells showed no immunoreactivity at this stage (Fig. 3C). Only a few clusters of cells showed some immuno-

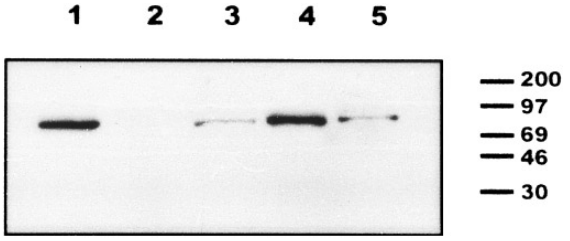


FIG. 2. Western blot analysis of total protein lysates from 13 dpc (lane 2) and 17 (lane 3) dpc embryo limbs or neonatal (lane 4) and adult (lane 5) skeletal muscle. The blots were incubated with the affinity-purified site-specific anti-PKCθ antiserum. Total protein lysate from overexpressing PKCθ COS1 cells (lane 1) was used as positive control. Fifty micrograms of protein lysate was loaded into each lane. The specificity of the band was determined by electrophoretic mobility and competition using an excess of the specific immunogen peptide (not shown).

reactivity and these cells may be precursors of fetal muscle (see below). However, few myosin-positive primary fibers also show some immunoreactivity. At 17 dpc a sarcoplasmic fluorescence was evident in most of the muscle fibers (Fig. 3D), with smaller secondary fibers showing stronger staining. A few groups of fibers were devoid of immunoreactivity.

To investigate whether this late onset of PKCθ expression was unique for this isoform, we analyzed the expression patterns of eight additional isoforms (PKC α, β_1, β_2, γ, δ, ϵ, ζ, and η), in embryonic and fetal limb muscle, by Western blot (Fig. 4). The results showed that PKC γ and ζ are expressed at both 13 and 17 dpc, while PKC α, β_1, β_2, and η are only expressed at 13 but not 17 dpc. PKC δ and ϵ were not present at either developmental stage. Since PKCθ is expressed at 17 dpc and at older ages, it is unique among the isoforms tested, in that it is selectively expressed in fetal muscle.

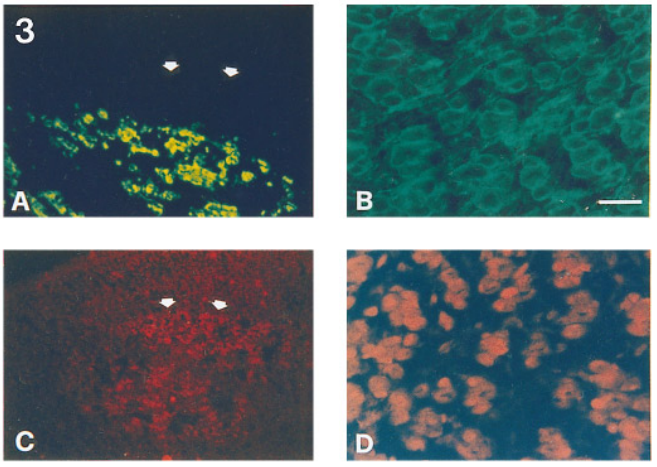
PKCθ Is Not Expressed in Embryonic Myoblasts in Culture

To verify whether the differential expression of PKCθ during limb muscle development could also be detected in different myogenic cells, primary cultures of embryonic (from 11 dpc limbs) and fetal (from 16 dpc limbs) myoblasts were analyzed, both during the proliferative phase and after myotube formation (Cusella De Angelis *et al.*, 1994). Immunofluorescence analysis shown in Fig. 5 revealed that neither embryonic myoblasts nor the myotubes they form in culture have detectable levels of PKCθ (Figs. 5A and 5D). By contrast, both fetal myoblasts and satellite cells express high levels of PKCθ during the proliferative period (Figs. 5B and 5C). After differentiation, myotubes derived from these cells continue to express PKCθ, although at an apparently lower level (Figs. 5E and 5F). These results indicate that the expression of PKCθ is specific for fetal and adult myogenic cells and that it is independent of their differentiation state.

The Ectopic Expression of PKCθ in Embryonic Myoblasts Induces Sensitivity to TGFβ

Since PKCs are usually involved in signal transduction, we examined whether the differential expression of PKCθ in embryonic and fetal myogenic cells may be causally related to their differential sensitivity to extracellular factors which interfere with differentiation such as TGFβ and phorbol esters. For this purpose we transfected embryonic myoblasts with an expression vector containing the PKCθ full-length cDNA and then exposed the transfected cells to TGFβ (5 ng/ml) or TPA (100 n*M*). After 2 days in culture the cells were fixed and the PKCθ-overexpressing cells (which could be easily identified by staining with anti-PKCθ antiserum) were analyzed for myosin expression under the various experimental conditions. Overexpression of PKCθ did not alter differentiation per se, since roughly the same proportion of transfected and nontransfected cells differentiated under the culture conditions employed (not shown). However, in the presence of either TGFβ or TPA, differentiation of PKCθ-overexpressing cells decreased dramatically to less than 10% of controls (Fig. 6). The nontransfected cells were not affected by the TGFβ or TPA treatment (not shown), in agreement with previous reports (Cusella-De Angelis *et al.*, 1994). Figure 7 shows an example of a PKCθ-overexpressing cell which is not myosin positive in the presence of TGFβ (Figs. 7B and 7D) while PKCθ-overexpressing cells also express skeletal myosin in untreated cultures (Figs. 7A and 7C). Thus, overexpression of PKCθ makes embryonic myoblasts sensitive to both TGFβ and TPA.

Although it appears that PKCθ can regulate myoblast diversification, it is possible that overexpression of any isoform of PKC would produce these same changes. To test this possibility, embryonic myoblasts were transfected with the expression vectors containing the full-length cDNAs coding for PKCα, δ, and ζ and then treated with TGFβ exactly as described above. The results (Fig. 6) show that overexpression of PKCα and ζ neither inhibits differentiation per se nor manifests sensitivity to TGFβ. Surprisingly, in two independent experiments, we did not detect any PKCδ-overexpressing cells in either control or treated cultures. Figure 7 shows an example of a PKCα-overexpressing cell which differentiates in the presence of TGFβ (Figs. 7F and 7H), as in control cultures (Figs. 7E and 7G). Thus, TGFβ-stimulated inhibition of myoblast differentiation correlates specifically with the presence of PKCθ in myogenic cells. In contrast, when treated with TPA, PKCα-overexpressing cells do not differentiate (Fig. 6). These data show that more than one PKC isoform can mediate the TPA inhibitory effect on differentiation. For this reason, it appears that the phorbol ester and the TGFβ effects on differentiation may occur through separate mechanisms.



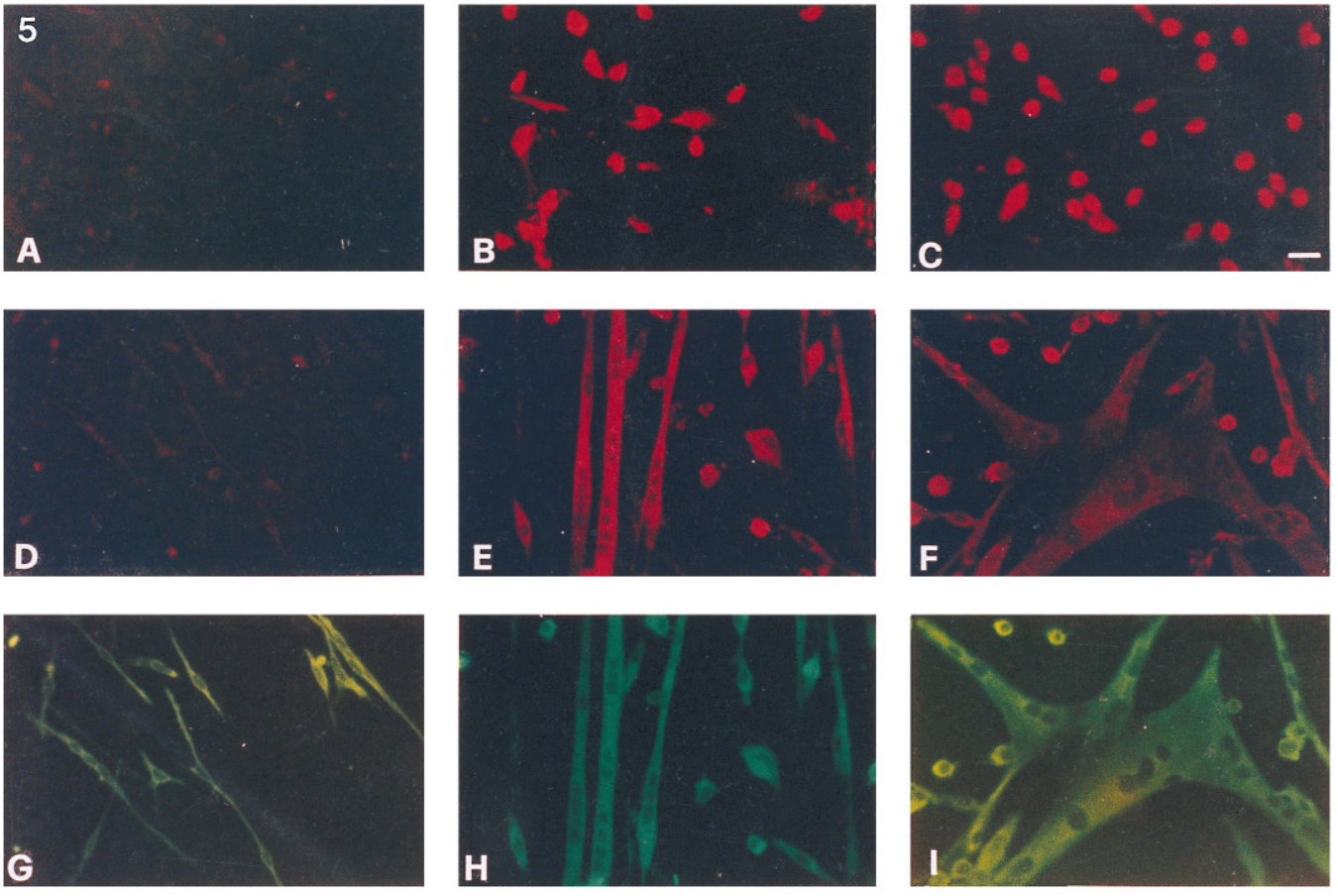


FIG. 3. Double-immunofluorescence analysis of 10-μm cryosections of 13 dpc (A, C) and 17 dpc (B, D) embryo limbs incubated with the anti-skeletal myosin monoclonal antibody (mAb) MF20 (A, B) and with anti-PKCθ antiserum (C, D). Arrows indicate a cluster of PKCθ-positive cells, which are myosin negative in the 13 dpc embryonic limb. Bar: 10 μm.

FIG. 5. Double-immunofluorescence analysis of embryonic (A, D, G) and fetal (B, E, H) myoblasts and satellite cells (C, F, I), under proliferating (A–C) and differentiating (D–I) conditions, Days 1 and 3, respectively. Fixed cells were incubated with the anti-PKCθ antiserum (A–F) and the anti-skeletal myosin mAb MF20 (G–I). Bar: 10 μm.

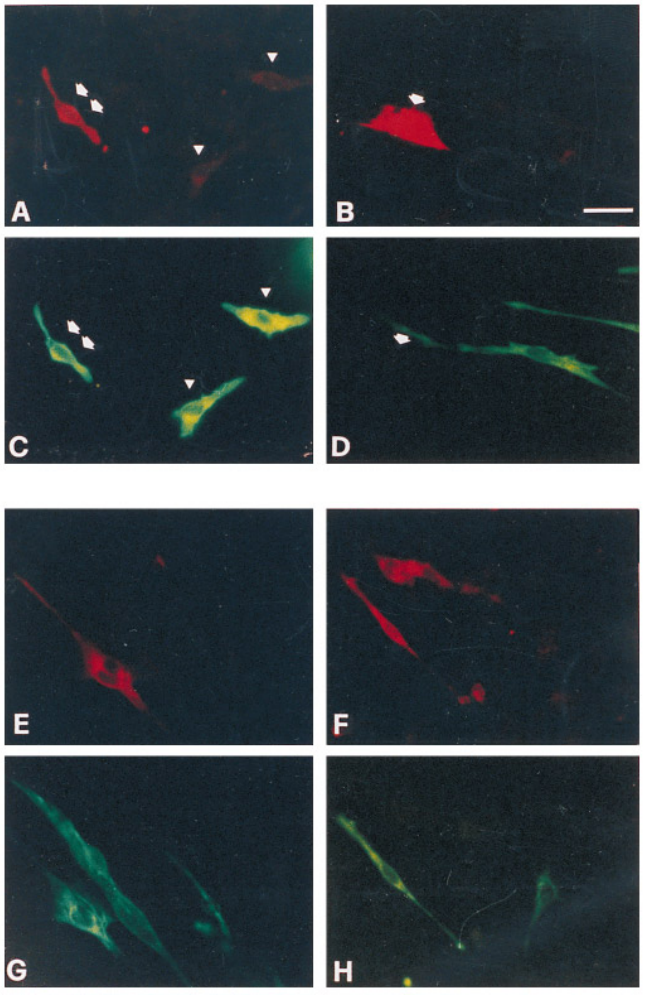


FIG. 7. Double-immunofluorescence analysis of embryonic limb cells transfected with the PKCθ (A–D) or PKCα (E–H) expression vectors and cultured in the absence (A, C, E, G) or in the presence (B, D, F, H) of 5 ng/ml TGFβ. The cells were incubated with the anti-PKCθ specific antiserum (A, B) or with the anti-PKCα specific antiserum (E, F) and the anti-skeletal myosin mAb MF20 (C, D, G, H). The single arrow indicates one PKCθ-overexpressing embryonic limb cell which is not myosin-positive in TGFβ-treated culture; two arrows indicate one PKCθ-overexpressing myosin-positive embryonic myocyte in untreated culture; arrowheads indicate myosin-positive embryonic myocytes which are PKCθ-negative. Bar: 10 μm.

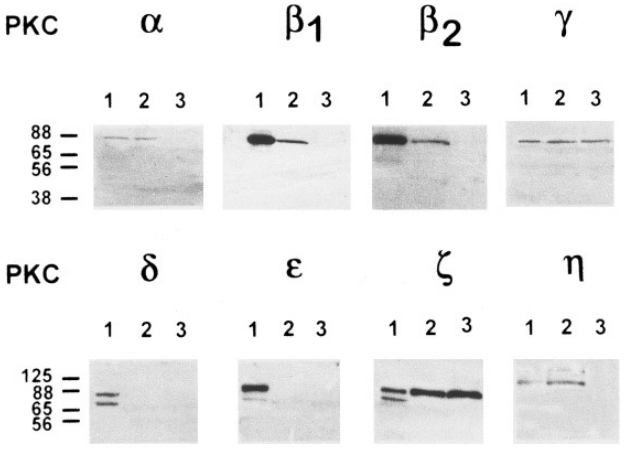


FIG. 4. Western blot using total protein lysates from 13 dpc (lane 2) and 17 dpc (lane 3) embryo limbs, for eight different PKC isoforms. Total lysate from brain (lane 1) was used as positive control. Fifty micrograms of protein lysate was loaded into each lane. The specificity of the bands was determined by electrophoretic mobility and competition using an excess of the specific immunogen peptide (not shown).

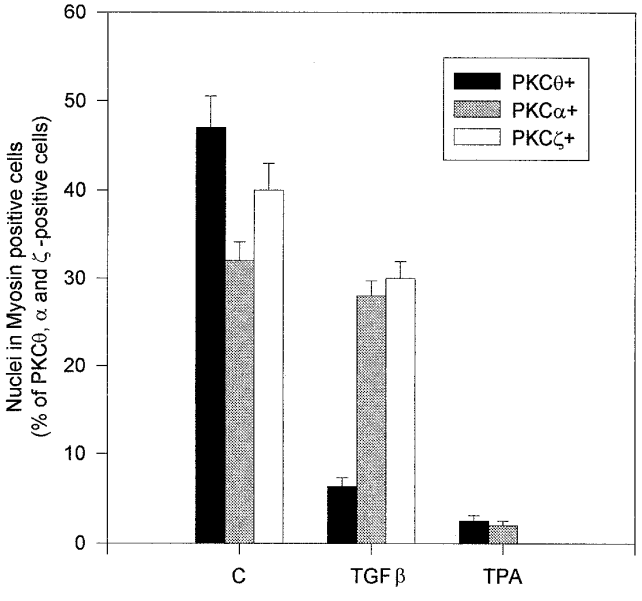


FIG. 6. Effect of TGFβ or TPA treatment on differentiation of embryonic myoblasts overexpressing specific PKC isoforms, as indicated. Primary embryonic limb cell cultures were transiently transfected with the specific expression plasmids, in the absence (C) or in the presence of TGFβ (5 ng/ml) or TPA (100 n*M*), as indicated. After 2 days in culture, the percentage of nuclei of PKC-overexpressing cells which coexpress skeletal myosin was determined under each condition.

DISCUSSION

The data reported in this paper show that the differential sensitivity of embryonic and fetal myoblasts to TGFβ is dependent upon selective expression of the θ isoform of protein kinase C. This is the first evidence showing that the expression of a molecule involved in signal transduction is causally related to the phenotypic difference among dif-

ferent populations of myogenic cells. We suggest that this phenotypic difference is essential for asynchronous generation of muscle fibers during embryogenesis. Our data also show that the θ isoform of PKC is a downstream target in postreceptorial signaling induced by TGFβ in myogenic cells.

PKCθ has been initially identified as a muscle-specific isoform (Osada *et al.*, 1992). While subsequent studies have demonstrated its presence in other tissues (Wang *et al.*, 1993), PKCθ is the most abundant PKC isoform to be expressed in adult skeletal muscle. The data reported in this paper show that PKCθ is also expressed in neonatal and fetal but not in embryonic muscle *in vivo*. In addition, culture studies reveal that PKCθ is selectively expressed by fetal myoblasts, which *in vivo* populate the developing limb and body wall. No other PKC isoform, among those we have tested, shows the same pattern of expression. Indeed, no other isoform of enzymes involved in signal transduction is known to be expressed in fetal but not in embryonic myogenic cells. This fact raises the possibility that such differential expression may be functionally relevant to muscle histogenesis. Embryonic myoblasts, which do not express PKCθ, differentiate in the presence of TGFβ or TPA, an activator of certain PKC isoforms (cPKC and nPKC). However, when PKCθ is overexpressed in embryonic muscle cells, they become responsive to TGFβ and to TPA. PKCθ appears to specifically provide the sensitivity to TGFβ, since overexpression of two different isoforms, PKCα and ζ, is not effective. These latter data exclude the possibility that the observed PKCθ effect can be mimicked by overexpression of any PKC isoform, which may alter the delicate balance among the various isoforms, and thereby lead to a nonspecific response to extracellular signaling.

A different situation arises for the TPA-responsive pathway. Overexpression of PKCα was as effective as PKCθ in inducing sensitivity of embryonic myoblasts to TPA. Since most of the PKCs (classical and novel) are known to be direct targets for TPA, this result suggests that these different activated PKCs may serve to conjointly orchestrate differentiation in the myoblast. Whether these same conditions would be encountered in the normal physiological state is unclear. Nonetheless, our data indicate that the TGFβ signaling pathways are specifically mediated by PKCθ in myogenic cells. This hypothesis is also reinforced by the observation that different myogenic populations do not respond similarly to both factors. For example, satellite cells express a high level of PKCθ and are sensitive to TGFβ (Allen and Boxhorn, 1987); however, these cells are not responsive to TPA (Cossu *et al.*, 1983).

Embryonic and fetal myoblasts give rise *in vitro*, and probably *in vivo*, to myotubes which express different muscle-specific gene products, such as slow myosin heavy chain, muscle creatine kinase, and β-enolase (Barbieri *et al.*, 1990; Cusella-De Angelis *et al.*, 1994). It would be interesting, therefore, to know whether differential expression of PKCθ is responsible for both differential sensitivity to TGFβ and differential gene expression in fetal and embryonic myotubes. Thus far, unavailability of specific antibodies has precluded the single cell analysis, which is necessary given that embryonic and fetal primary myogenic cells cannot be selected after transfection.

Why differential expression of PKCθ should dictate the differential fate of embryonic versus fetal myoblasts remains, at present, a matter of speculation. It is a fact, however, that only a fraction of the total myogenic precursor population differentiates in the embryo to give rise to primary fibers. A possible mechanism to ensure that certain myoblasts will differentiate in an environment which is permissive for proliferation may be based on unresponsiveness of these myoblasts to growth factors and/or to molecules which inhibit differentiation. A long search for growth factors which may affect selectively proliferation of fetal versus embryonic cells failed to yield any possible candidate molecule for such a role. Recently, loss of fibroblast growth factor receptor has been proposed as a possible mechanism to control myoblast differentiation *in vivo* (Itoh *et al.*, 1996). However, this does not explain why certain myoblasts differentiate and others do not. On the other hand, TGFβ became an obvious candidate for this role when it was shown that it can inhibit differentiation of fetal but not of embryonic cells (Cusella-De Angelis *et al.*, 1994). Furthermore, the limb bud contains high levels of TGFβ in the place and at the time when differentiation of embryonic myoblasts occurs (Millan *et al.*, 1991; Schmid *et al.*, 1991). It is worth noting that muscle differentiation begins in the proximal region of the limb. This is the region most distant from the apical ridge, which produces FGFs (Niswander and Martin, 1992; Drucker and Golfarb, 1993). Therefore, although the actual concentration of any single FGF in this region is very difficult to measure, it is conceivable that all myoblasts in this area may be exposed to a low growth factor concentration (or may down-regulate receptors) but to a high concentration of TGFβ. At this time, embryonic myoblasts, which do not express PKCθ and, thus, are insensitive to TGFβ, may differentiate into primary fibers, while differentiation of fetal myoblasts, which do express PKCθ, should be prevented by TGFβ. Once primary fibers are formed, they begin to produce growth factors such as FGF (Drucker and Golfarb, 1993) and, thus, promote a new wave of proliferation in fetal cells (De Angelis *et al.*, 1992). Furthermore, this hypothesis requires that when fetal myoblasts differentiate, they either become resistant to TGFβ (e.g., PKCθ negative) or the actual concentration of the various TGFβs decreases significantly. The first possibility is ruled out by our present data showing that primary fibers, after formation, begin to express PKCθ, while both fetal myoblasts and the secondary fibers they form express PKCθ. The second possibility is, rather, more likely, since our unpublished results show a dramatic decrease in the level of TGFβ1 message, just at the onset of fetal myoblast differentiation.

The identification of PKCθ as a key component of this pathway permits the design of experiments where expression of this enzyme *in vivo*, driven by the promoter of genes

expressed early in the limb bud, should provide each cell with PKCθ. If the prediction is correct, all myogenic cells should become sensitive to TGFβ, and primary fibers should not be formed in the mouse developing limbs. This possibility is currently under investigation.

ACKNOWLEDGMENTS

We are indebted to Dr. D. A. Fischman for the gift of the MF20 antibody. We thank Dr. M. Buckingham at Pasteur Institute for continuous discussion and critical reading of the manuscript and Drs. M. I. Senni and C. Sonnino for the excellent assistance. This work was funded by Grants from Telethon (245 to M.B. and A53 to G.C.), MURST (to G.C. and M.M.), P.F. ACRO-CNR and AIRC (to M.M.), and EC No. B104-CT95-0228 (to G.C).

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Received for publication April 1, 1996
Accepted August 26, 1996